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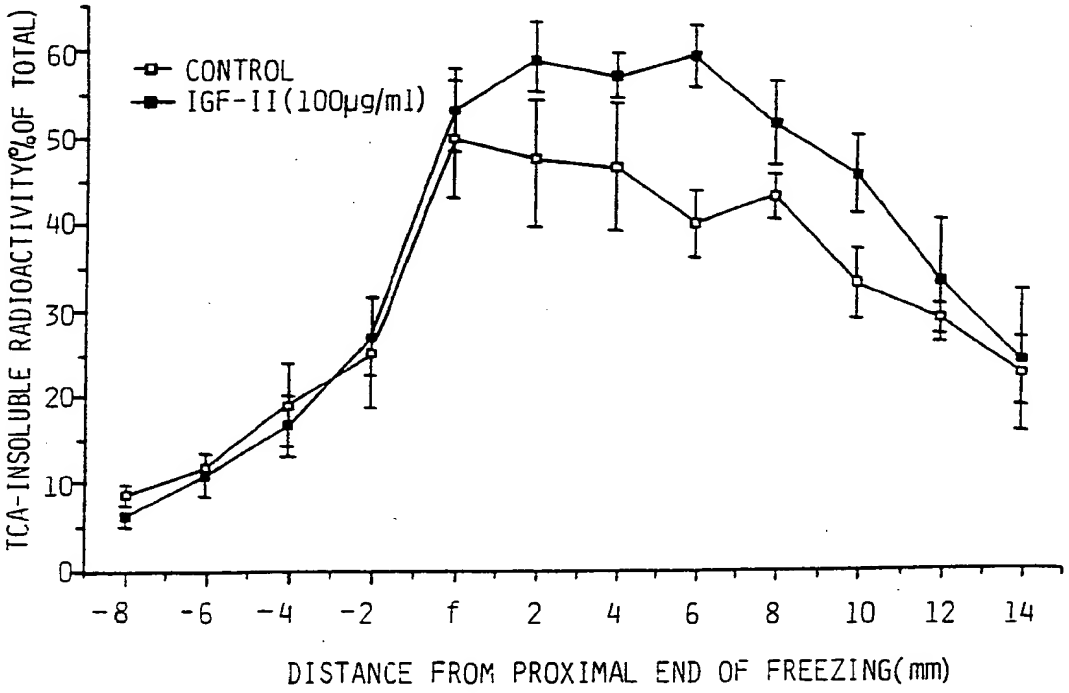

(57) Abstract

The use of IGF-II for preparing a medicament for nerve regeneration; and a method for medical treatment resulting in nerve regeneration, comprising the step of administering to patient in need of such treatment an effective amount of IGF-II.

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New medicinal use

The present invention relates to a new medicinal use, more particularly the use of insulin-like growth factor II (IGF-II) for preparing a medicament for nerve repair and regeneration.

The nervous system of a vertebrate is subdivided into the central nervous system (CNS), which consists of the brain and the spinal cord, and the peripheral nervous system (PNS), which includes the cranial and spinal nerves and the peripheral components of the autonomic nervous system. The present invention involves new techniques enabling improved nerve regeneration and the invention is applicable to disorders or damages in the central nervous system (CNS) and the peripheral nervous system (PNS).

Insulin-like growth factor II (IGF-II) is structurally significantly different from IGF-I and little is known about its therapeutic usefulness. IGF-II is a single-chain peptide containing 67 amino acid residues. For further details concerning IGF-II see Eur.J.Biochem. 190, 445-462 (1990), René E. Humbel, Review Insulin-like Growth Factors I and II. IGF--II and IGF-I acts via different receptors. The receptor for IGF-II is a single chain receptor with a molecular weight of 260 kD and it is identical with the mannose-6-phosphate receptor (Morgan et al. Nature 329, 301-307 (1987); Kiess et al., J.Biol.Chem. 263, 9339-9344 (988). The IGF-II receptor has no homology with the IGF-I receptor which is dimeric and shows protein kinase activity. IGF-II may crossreact with th IGF-I receptor but IGF-I does not crossreact with the IGF-II receptor (Roth, R.A. Science 1988, 239, 1269-1271).

It is known that growth factors such as IGF-I, FGF and NGF promote neurite extension in cultured dorsal root ganglia cells or neuroblastoma cells [Recio-Pinto, E. t al. J.Neurosci. 6, 1211-1219 (1986); Morrison, R.S. et al. Proc.Natl-.Acad.Sci. USA, 83, 7533-7541 (1986); Shelton, D.L., Proc-

.Natl.Acad.Sci, USA, 83, 2714-2718 (1986)]. Howev r, it is to be noted that growth of axons in a regenerating peripheral nerve is a different process with respect to the influence of growth factors and drugs. Thus, regeneration of the sciatic nerve is unaffected by NGF [Rich, K.M., J. Comparative Neurology 230, 110-118 (1984)] and a variety of drugs used to induce neurite formation in culture. It also known that IGF-I stimulates nerve regeneration in vivo in rat. (Regeneration at the rat sciatic nerve, Dissertation Departement of Zoophysiology, University of Lund, Sweden, February 9, 1990, by Jacob Sjöberg).

In vitro data:

IGF-II has been demonstrated to stimulate neurite outgrowth and survival in vitro of cultured chick sympathetic and sensory neurons [Recio-Pinto E. et al., J.Neurosoci. 6, 1211-1219 (1986); Bothwell, J.Neurosci. Res 225-231 (1982)].

However, it could not be concluded from these results that IGF-II would also stimulate nerve regeneration in vivo, since eg. NGF is clearly effective in vitro but has no effect on nerve regneration in vivo. Furthermore, our results on nerve explants surprisingly showed that IGF-II affected the nerve-associated cells rather than the neurons themselves.

In connection with extensive research and experimentation it has now surprisingly been found that IGF-II effectively promotes nerve regeneration, albeit through a different mechanism than IGF-I, as will be shown below in the experimental section of this disclosure.

Administration of IGF-II in vivo showed that it significantly improved regneration of rat sciatic nerves subjected to a freeze lesion. Measurments of thymidine incorporation along the nerve following such administration suggested that IGF-II acted by stimulation of proliferation of nerve associated cells. This implication was further substantiated by experiments in culture which showed that IGF-II enhanced proliferation of nerve associated cells in cultured segments of the sciatic nerve. The latter experiments also demonstrated

that IGF-II acts via different mechanisms than IGF-I which fails to affect proliferation in excised segments of the sciatic nerve.

The claims made in this application are primarily based on our finding that IGF-II stimulates regeneration of the sciatic nerve in adult rats. This stimulation appears to occur through activation of nerve associated cells. The findings, with respect to the effects on regneration and mechanism of action are new and surprising and affords new curative techniques for the treatment of damaged nervous tissue.

Accordingly, it is a main object of the present invention to provide new techniques for preparing medicaments effective in providing improved regeneration of nerves subjected to damage.

Another object of the invention is to provide for a medicament useful in the treatment of neuropathy.

A third object of the invention is to provide techniques for preparing a medicament for use in the treatment of degenerative neural disorders.

Yet another object is to provide techniques for preparing medicaments, wherein IGF-II is used in combination with IGF-I.

Accordingly, the invention resides in the use of IGF-II for preparing a medicament useful for nerve regeneration. According to preferred aspects of the invention such medicament can be used in the treatment of neuropathy or, alternatively, in the treatment of degenerative neural disorders and survival of nervous tissue for transplantation.

The invention also resides in a method for medical treatment resulting in nerve regeneration, said method comprising the step of administering to a patient in need of treatment for nerve regeneration an effective amount of IGF-II.

In this method IGF-II can be used in combination with IGF-I. The mechanisms of action between IGF-I and IGF-II are different. IGF-I stimulates the neuronal cells and IGF-II

stimulates th nerve associated cells whereby a combination of the two gives an optimal effect of regeneration. The proportions between these two growth factors may vary between 1:10 to 10:1 based on weight.

The medicament used in accord with the present invention may thus in accordance with traditional pharmaceutical practice be formulated for use in human or veterinary medicine for therapeutic purposes. The compositions may include the active ingredient IGF-II in combination with a pharmaceutically or diganostically acceptable carrier, which may be solid, semisolid or liquid.

The compositions include those in a form adapted for topical application.

Suitable forms of the composition of this invention include tablets, capsules, sirups, suspensions, solutions, and forms suitable for injection or infusion. Such compositions may contain conventional pharmaceutically acceptable materials, such as diluents, binders, colours, flavours, preservatives, disintegrants and the like in accordance with conventional pharmaceutical practice in the manner well understood by those skilled in the art of formulating drugs.

Injectable or infusable compositions of IGF-II are particularly suitable as increased levels of IGF-II in the circulation can occur after administration by injection or infusion.

The invention will in the following be described by non-limiting examples with reference to the appended drawings, wherein:

Figure 1 is a diagram illustrating experiments involving effect of IGF-I at various concentrations on <u>in vitro</u> proliferation in the rat sciatic nerve;

Figure 2 is a corresponding diagram but using IGF-II instead of IGF-I;

Figure 3 shows a diagram on thymidine incorporation in freeze-injured rat sciatic nerve with and without treatment with IGF-II.

Figure 4 shows a diagram on regeneration distance in freeze-injured rat sciatic nerve with and without treatment with IGF-II; and

Fig. 5 shows a corresponding diagram comparing the effect of regeneration using two different concentrations of IGF-II.

EXAMPLE 1

Comparison between IGF-I and IGF-II in the rat sciatic nerve in vitro.

Animals

Female Sprague-Dawley rats weighing around 200 g were used. The rats were anaesthetized by an intraperitoneal injection of 0.3 ml of a 1:1.2 mixture of pentobarbital (60 mg/ml) saline (0.9%) and diazepam 5 mg/ml). Both sciatic nerves were then removed by dissection.

Culture

The dissected nerve was cut into 4 mm segments. These were transferred to plastic Petri dishes (Nunclon 3.5 cm) containing serum free RPMI 1640 medium without or with IGF-I or IGF-II at concentrations indicated in Fig. 1 and Fig. 2, respectively. Each dish was supplied with 3-4 nerve segments. The preparations were incubated for 48 h at 37°C in a humidified atmosphere of 5% CO_2 in air.

Thymidine incorporation

Thymidine incorporation was measured essentially as described previously (J. Sjöberg & M. Kanje, Brain Research, 530 (1990) 167-169). The cultured segments were rinsed in rat Ringer solution and transferred to test tubes containing Ringer solution supplied with 50 µCi ^{3}H-thymidine/ml (Amersham, specific activity 91 Ci/mmol). The tubes were incubated in a waterbath at 37°C for 2h. The segments were then washed in ice-cold Ringer solution and subsequently extracted in 10% tri-

chloro acetic acid (TCA) at 4°C for a minimum of 2h. The segments were then washed in TCA and finally dissolved Soluene 350 over night. Radioactivity in the TCA-soluble and insoluble fractions was determined by liquid scintillation counting. Thymidine incorporation was expressed as TCA-insoluble radioactivity in percent of total radioactivity in the segment.

The results of the experiments on thymidine incorporation at varying concentrations of the respective growth factors are illustrated in Figs. 1 and 2.

Fig. 1 shows the results of IGF-I at varying concentrations and clearly shows that IGF-I at concentrations varying from 0.01 to 1 ng/ml has no significant effect on the proliferation in the sciatic nerve *in vitro*, while an inhibition was observed at 100 ng as compared to a control devoid of growth factor.

Fig. 2 illustrates a corresponding set of experiments using IGF-II and Fig. 2 clearly illustrates a significant increase of the *in vitro* proliferation in the interval between 0.1 and 1000 ng/ml. This is accordingly also an indication of the fact that IGF-II affects nerve growth by a different mechanism than does IGF-I.

EXAMPLE 2

The stimulatory effect of IGF-II on regeneration of freeze-injured rat sciatic nerve.

Animals

Female Sprague-Dawley rats (Alab, Sweden) weighing approximately 200 g were anesthetized by i.p. injection of 0.35 ml of a Nembutal (50 mg/ml)-Valium (5 mg/ml)-saline mixture (1:2:1 v/v). The sciatic nerve was exposed in the mid-thigh and frozen over a 15 mm segment with specially designed tweezers chilled in liquid nitrogen. Freezing was performed twice for 30 s with 2 min thawing in between. The proximal end of the frozen segment was labeled by attaching a 9-0 suture to the epineurium.

An osmotic minipump (model 2001, Alzet, USA) filled with a modified Ringer solution (in mM): NaCL 139, KCl 2.4, $MgSO_4$ 2, NaH_2PO_4 0.6, Na_2HPO_4 3.25, pH 7.4 and different concentrations of IGF-1 (Kabi Pharmacia AB, Stockholm, Sweden) were implanted subcutaneously on the abdomen of the rat. The pump rate was 1.05 µl/h. From the pump a thin silicone catheter was drawn subcutaneously to the sciatic nerve. The catheter was sutured alongside the freeze-injured nerve with the catheter opening half-way down the freeze-injured segment (Fig.-1). The part of the catheter lying parallel to the nerve was perforated to increase the perfused area. Two animals were kept in each cage at a room temperature of 21°C and 52% humidity, and given water and food ad libitum.

EXAMPLE 3

Thymidine incorporation

The animals were sacrificed 6 days after surgery and the sciatic nerves were removed. The nerves were desheathed and incubated for 2h in vials containing 50 µCi of [^{3}H]thymidine (91 Ci/mmol) in 1 ml of modified Ringer solution at 37°C. After thorough washing with ice-cold Ringer solution the nerves were cut in 2 mm pieces and extracted for 2x30 min in 10% trichloroacetic acid (TCA). TCA-insoluble material was solubilized in 200 µl Soluene 350 (Packard). Radioactivity of TCA-soluble and insoluble fractions were determined by liquid scintillation counting. Thymidine incorporation was measured in the same way as in Example 1 esssentially according to the techniques described in the Sjöberg et al. Brain Research, 530 (1990) 167-169.

Fig. 3 shows a diagram illustrating the effect of IGF-II on the incorporation of thymidine along the nerve 6 days after freezing. Comparison is made with a control using no IGF-II. As can be seen from the diagram IGF-II perfusion results in a significant increase in proliferation compared to the control with no IGF-II perfusion. This *in vivo* experiment thus illustrates with clarity the usefulness of IGF-II in

enhancing proliferation of regenerating nerve upon injury or other nerve cell disorder.

EXAMPLE 4

Example 2 was repeated and regeneration distances after 6 days profusion of the freeze-injured nerve section with and without administration of IGF-II and at two different concentrations of IGF-II are shown in Fig. 4 and Fig. 5, respectively.

As seen from the results illustrated in Fig. 4 treatment with IGF-II at 100 µg/ml results in a significant increase in regeneration distance as compared to the control.

Fig. 5 shows the dose response when using IGF-II at 25 µg and 100 µg/ml. In this figure is expressed as the difference in regeneration distance compared to the control. In Figs. 4 and 5 the asterisc means $p < 0.05$.

CLAIMS

1. The use of IGF-II for preparing a medicament for nerve regeneration through activation of nerve associated cells.

2. The use according to claim 1 for preparing a medicament for use in the treatment of neuropathy.

3. The use according to claim 2 for preparing a medicament for use in the treatment of degenerative neural disorders.

4. The use according to any preceding claim, wherein IGF-II is used in combination with IGF-I.

5. A method for medical treatment resulting in nerve regeneration, comprising the step of administering to a patient in need of such treatment an effective amount of IGF--II.

6. A method according to claim 5 for the treatment of neuropathy.

7. A method according to claim 5 for the treatment of degenerative neural disorders.

8. A method according to claim 5 as applied to using IGF-II for maintenance or survival of transplants.

FIG.1

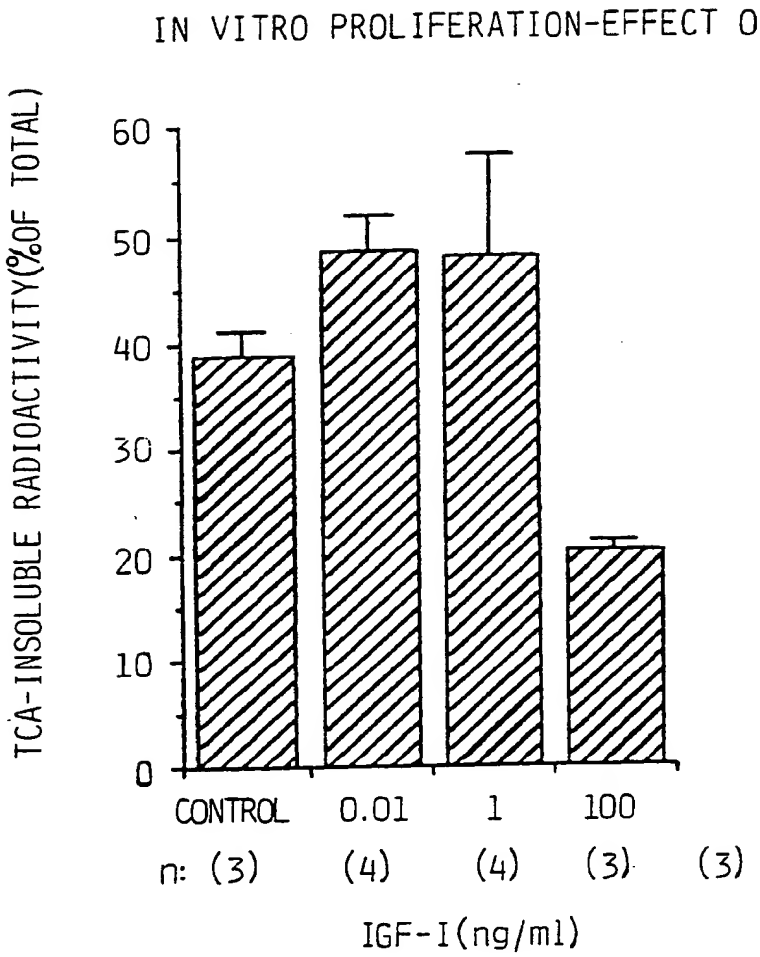


FIG.2

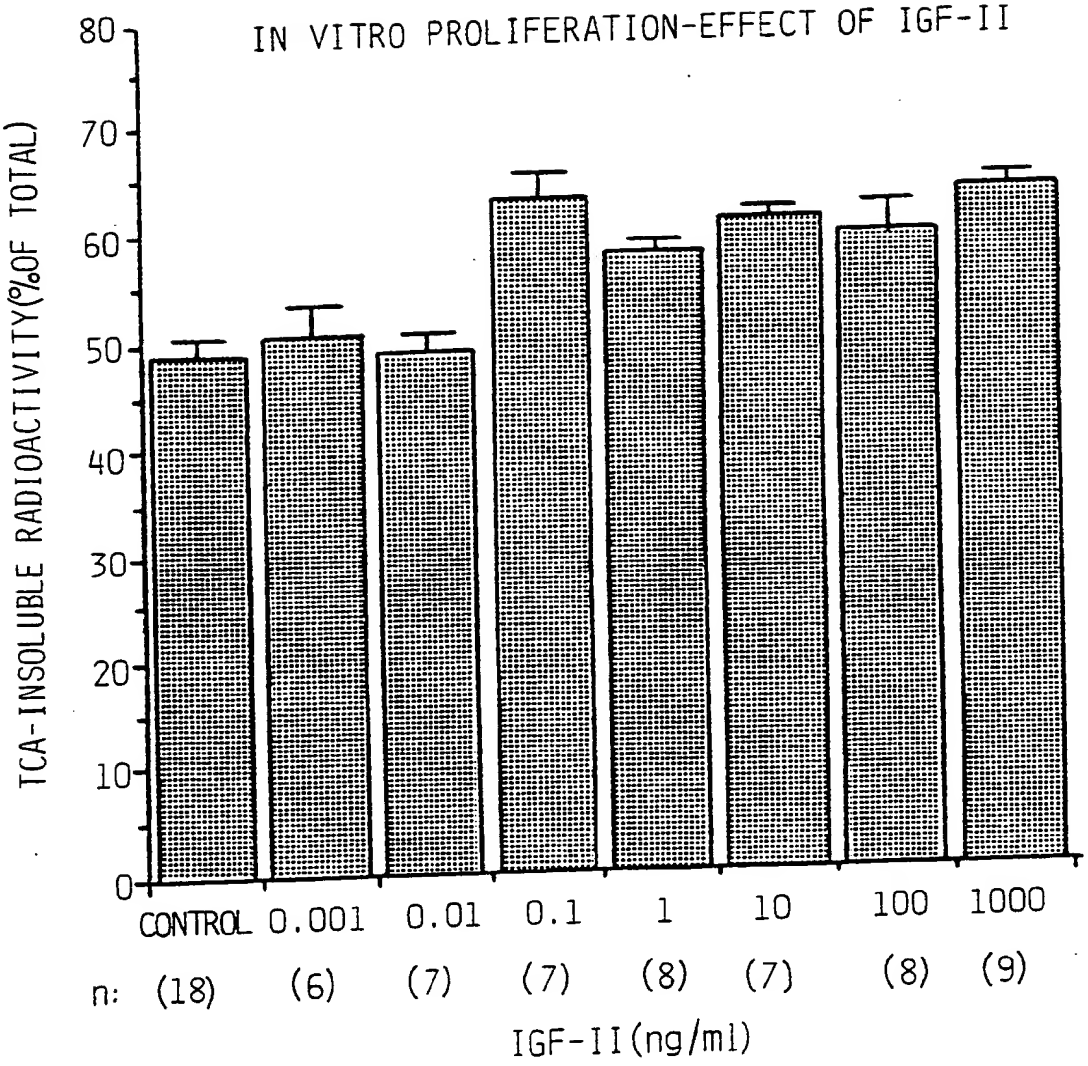


FIG.3

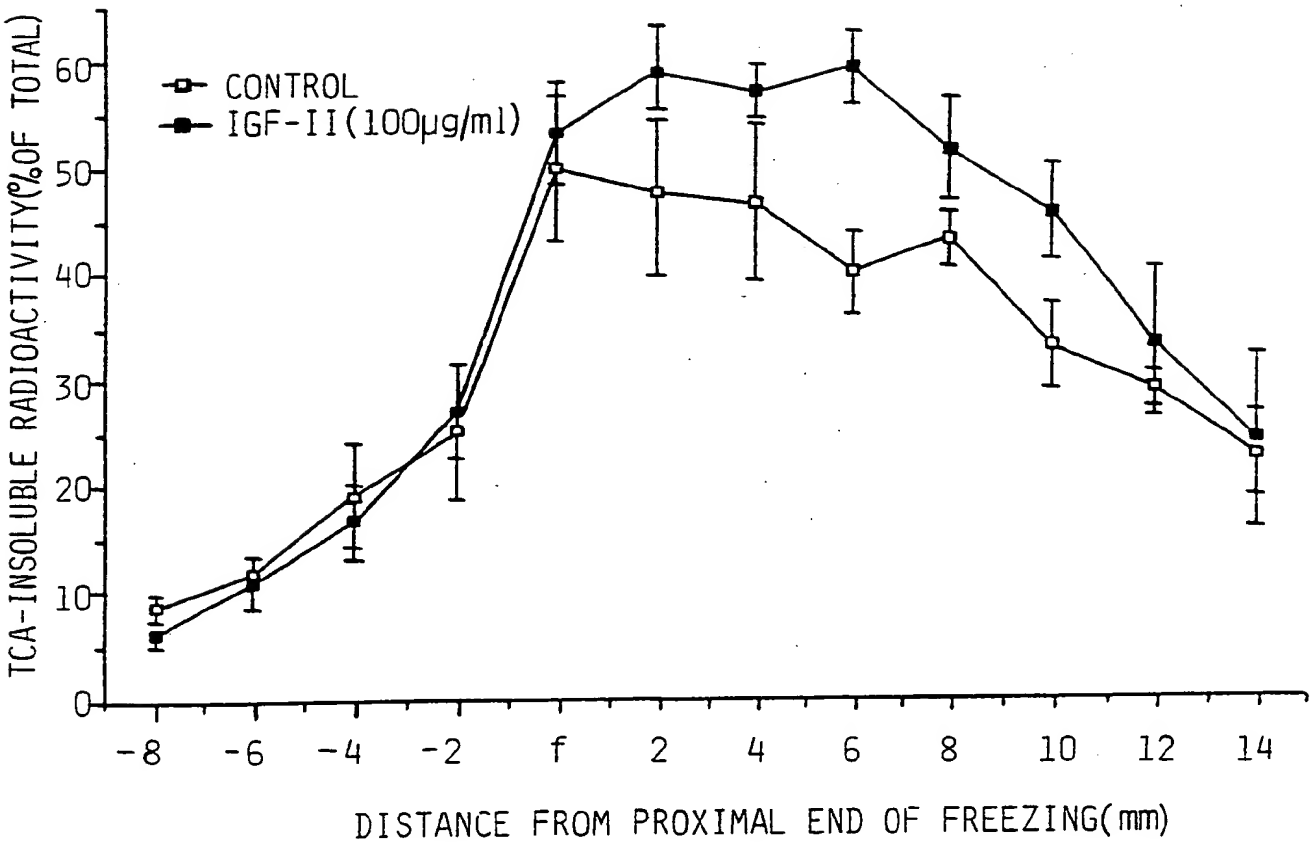


FIG.4

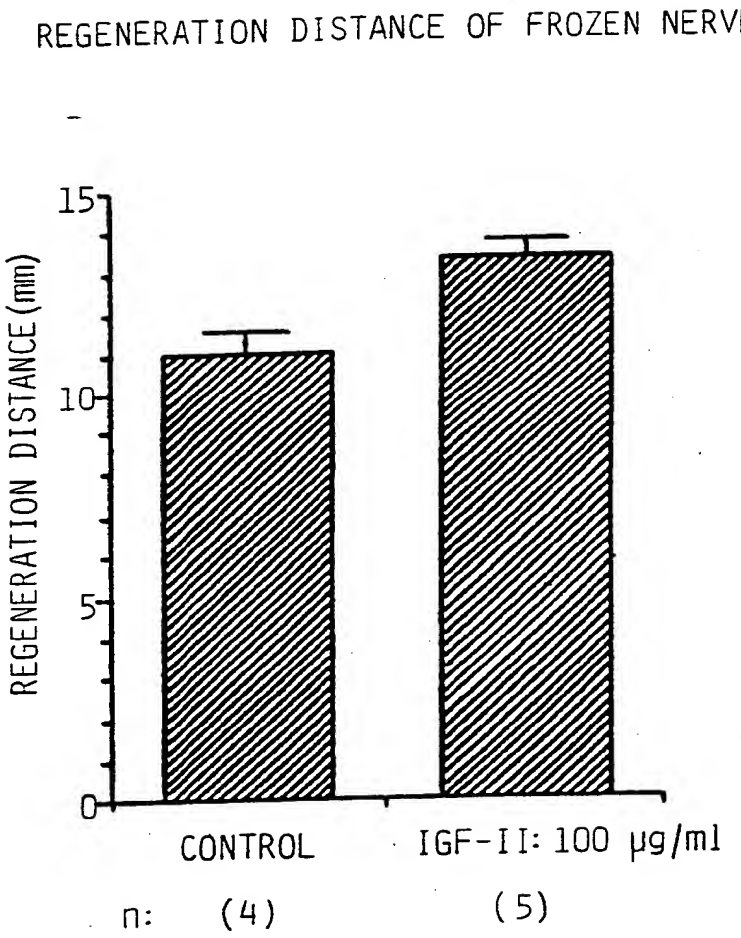
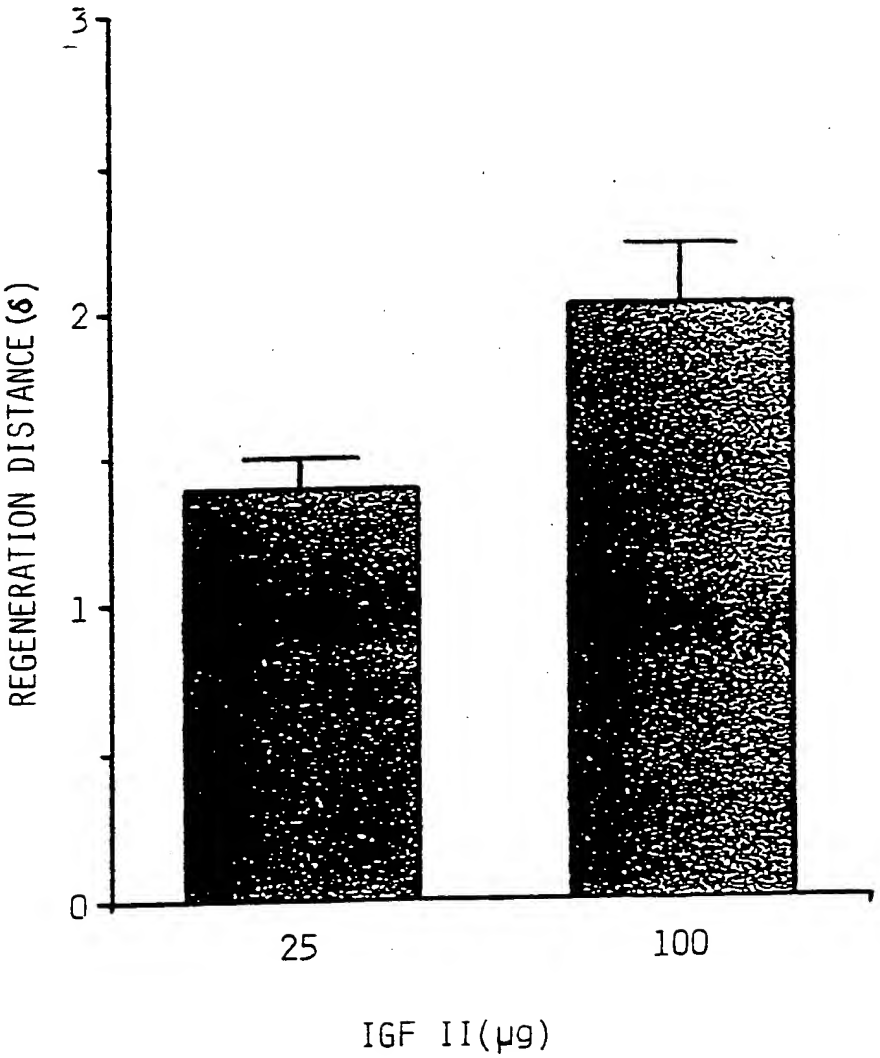


FIG.5

EFFECTS OF IGF-II ON REGENERATION OF THE RAT SCIATIC NERVE



INTERNATIONAL SEARCH REPORT

International Application No PCT/SE 92/00275

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC5: A 61 K 37/02

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
IPC5	A 61 K; C 07 K

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in Fields Searched[8]

SE,DK,FI,NO classes as above

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category *	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
X	WO, A1, 14838 (CEPHALON, INC.) 13 December 1990, see especially claims 1, 6 and 14 --	1-4
X	The Journal of Cell Biology, Vol. 110, April 1990 Caroni P. et al.: "Nerve sprouting in Innervated Adult Skeletal Muscle Induced by Exposure to Elevated Levels of Insulin-like Growth Factors", see page 1307 - page 1317 --	1-3
X	Soc. Neurosci. Abstr., Vol. 13, No. 2, 1987 Ishii, D. N.: "Insulin-like Growth Factor-II gene expression in muscle relationship to synapse elimination and nerve regeneration", see page 1211 --	1-3

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance, the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance, the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
6th August 1992	1992 -08- 12
International Searching Authority	**Signature of Authorized Officer**
SWEDISH PATENT OFFICE	Carolina Palmcrantz Carolina Palmcrantz

Form PCT/ISA/210 (second sheet) (January 1985)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
X	Brain Research, Vol. 302, 1984 Recio-Pinto, E. et al.: "Effects of Insulin, Insulin-like Growth Factor-II and Nerve Growth Factor on Neurite Outgrowth in Cultured Human Neuroblastoma Cells", see page 323 - page 334 --	1-3
X	The Journal of Neuroscience, Vol. 6, No. 5, May 1986 Recio-Pinto, E. et al.: "Effects of Insulin, Insulin-like Growth Factor-II, and NerveGrowth Factor on Neurite Formation and Survival in Cultured Sympathetic and Sensory Neurons", see page 1211 - page 1219 --	1-3
X	WO, A1, 9102067 (MAX PLANCK-GESELLSCHAFT ZUR FÖRDERUNG DER WISSENCHAFTEN E.V.) 21 February 1991, see especially claims 1, 5, 11 and 21 --	1-3
A	Dialog Information Services, file 5, BIOSIS 69-91, Dialog Acc.No. 7095055, Sjöberg J et al: "Insulin-like growth factor IGF-1 as a stimulator of regeneration in the freeze-injureo rat sciatic nerve", & Brain Res 485 (1), 1989, p102-108 --	1-4
A	Dialog Information Services, file 155, Medline 66-91, Dialog Acc.No. 05445532, Whitfield H.J. et al: "Isolation of a cDNA clone encoding rat isulin-like growth factor-II precursor", & Nature Nov 15-21 1984; 312 (5991) p277-80 --	1-4

FURTHER INFORMATION CONTINUED FR M THE SECOND SHEET

V. ☒ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [1]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers 5-8, because they relate to subject matter not required to be searched by this Authority, namely:

See PCT Rule 39.1(iv): Methods for treatment of the human or animal body by surgery or therapy, as well as diagnostic methods.

2. ☐ Claim numbers....., because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claim numbers....., because they are dependent claims and are not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [2]

This International Searching Authority found multiple inventions in this international application as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the the claims. It is covered by claim numbers:

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additonal fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional seach fees.

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. PCT/SE 92/00275

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the Swedish Patent Office EDP file on 01/07/92 The Swedish Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A1- 14838	90-12-13	NONE		
WO-A1- 9102067	91-02-21	EP-A-	0484416	92-05-13